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Effect of Liposomal Size on the Calorimetric Behavior of Mixed-Chain Phosphatidylcholine Bilayer Dispersions†

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ABSTRACT: The effect of liposomal size on the endothermic transition profiles of the saturated mixed-chain phosphatidylcholines 1-stearoyl-2-myristoyl-*sn*-glycero-3-phosphocholine and 1-stearoyl-2-caproyl-*sn*-glycero-3-phosphocholine has been investigated. Liposomal bilayer dispersions of progressively smaller average diameter were prepared by extrusion of coarse multilamellar preparations of these lipids through polycarbonate membrane filters of decreasing pore size from 3- to 0.2-μm diameter. These samples were then investigated by high-sensitivity differential scanning calorimetry and negative-stain electron microscopy. It was found that coarse dispersions of the above lipids are composed of liposomes whose average diameter is considerably smaller than that typically associated with multilamellar liposomes of synthetic phosphatidylcholines. This fact, coupled with an analysis of the size dependence of the transition cooperativity, leads to the conclusion that the small size of the liposomes limits the transition cooperativity in coarse dispersions of these mixed-chain phosphatidylcholines. Thus, the broad and highly asymmetric transition profiles that have been observed in previous studies of these phosphatidylcholines are postulated to arise largely from this size dependence, rather than from characteristic packing properties of the phospholipid acyl chains as has been previously suggested.

Recently, we reported on the calorimetric behavior of a series of saturated mixed-chain phosphatidylcholines whose *sn*-2 acyl chains were varied to be from two carbon atoms to eight carbon atoms shorter than the *sn*-1 acyl chains: C(18):C(16)-PC[1] to C(18):C(10)-PC (Mason et al., 1981a). This study revealed that in multilamellar bilayer dispersions derived from these phosphatidylcholines, the gel ↔ liquid-crystalline phase transition becomes broader, less cooperative, and generally of higher transition enthalpy as the chain length inequivalence is increased.

When high-sensitivity DSC was employed, it could also be seen that the transition profiles of C(18):C(14)-PC and C(18):C(10)-PC were composites of two or more individual transition peaks. A similar observation was made in a high-sensitivity DSC study of C(18):C(14)-PC and C(16):C(14)-PC by Chen & Sturtevant (1981). It has been suggested that these multiple peaks arise from the segregation of the bilayer into regions of interdigitated and random packing of the acyl chains (Chen & Sturtevant, 1981) or from the packing of the acyl chains into more than one interdigitated conformation within the gel state of these phosphatidylcholines (Mason et al., 1981a).

Here, we extend the previous studies by examining the effect of liposomal size on the endothermic transition profiles of C(18):C(14)-PC and C(18):C(10)-PC bilayer dispersions. Liposomal dispersions of progressively smaller average diameter were prepared by extrusion of coarse preparations through polycarbonate membrane filters of decreasing pore size from 3- to 0.2-μm diameter. These samples were then examined by negative-stain electron microscopy and high-sensitivity DSC.

It was found that coarse dispersions of C(18):C(14)-PC and C(18):C(10)-PC form liposomes whose average diameter is considerably smaller than that typically associated with synthetic phosphatidylcholine multilamellar liposomes such as dipalmitoylphosphatidylcholine. This fact, coupled with an examination of the liposomal size dependence of the transition cooperativity based upon the analysis introduced by Marsh et al. (1977), leads to the conclusion that the cooperativity in the thermal transitions of these phosphatidylcholines is largely limited by the size of the liposomes. Thus, the large transition breadth and appearance of multiple peaks that have been

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[1] Abbreviations: C(18):C(18)-PC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; C(18):C(16)-PC, 1-stearoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine; C(18):C(14)-PC, 1-stearoyl-2-myristoyl-*sn*-glycero-3-phosphocholine; C(16):C(14)-PC, 1-palmitoyl-2-myristoyl-*sn*-glycero-3-phosphocholine; C(18):C(10)-PC, 1-stearoyl-2-caproyl-*sn*-glycero-3-phosphocholine; C(16):C(16)-PC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance.

0006-2960/83/0422-2013$01.50/0

Table I: Thermodynamic Parameters of the Coarse and Sized Liposomal Dispersions[a]

dispersions	filter size (μm)	average radius (μm)	T_o (°C)	T_c (°C)	T_{m1} (°C)	T_{m2} (°C)	T_{m3} (°C)	$\Delta T_{1/2}$ (°C)	ΔH (kcal/mol)	R_{cl} (μm)	R_{cu} (μm)
C(18):C(14)-PC	disp	0.50	28.6	32.1	29.9	30.0		0.63	5.5	0.10	0.25
C(18):C(14)-PC	3.0		28.3	32.8	29.9	30.2		0.71	6.5	0.050	0.20
C(18):C(14)-PC	1.0	0.35	28.4	32.6	30.0	30.2		0.81	6.7	0.045	0.15
C(18):C(14)-PC	0.2	0.15	27.8	33.1		30.4		1.3	5.9	0.040	0.10
C(18):C(10)-PC	disp	0.30	17.4	21.0	19.6	19.9	20.2	0.91	9.7	0.10	0.20
C(18):C(10)-PC	3.0		17.7	20.9	19.4		19.9	0.74	9.9		
C(18):C(10)-PC	1.0	0.35	17.5	20.6	19.3	19.7	19.8	0.81	10.4		
C(18):C(10)-PC	0.2	0.15	16.3	20.1	19.2			0.68	12.2	0.030	0.10

[a] Table I lists the thermodynamic parameters derived from the calorimetric scans of Figures 2 and 3. Filter size refers to the polycarbonate membrane pore size used in the preparation of the indicated dispersions; disp refers to unfiltered coarse dispersions. Average radius is the mass average radius of the indicated dispersions as determined from the distribution profiles such as that shown in Figure 1. T_o and T_c are the onset and completion temperatures, respectively, of the thermal transitions. The transition temperatures (T_{m1}, T_{m2}, T_{m3}) indicate the maxima in the excess heat capacity profiles of the thermal transitions. ΔH is the transition enthalpy which is taken as the area under the transition curve. R_{cl} and R_{cu} are the lower and upper limits to the apparent critical radius, respectively, for the indicated dispersions. These values were calculated as described in the text.

observed in the transition profiles of these asymmetric phosphatidylcholines appear to arise more from this size dependence than from characteristic packing properties of the acyl chains.

Materials and Methods

Synthesis of Saturated Mixed-Chain Phosphatidylcholines. The method employed for the synthesis of the saturated mixed-chain phosphatidylcholines C(18):C(14)-PC and C(18):C(10)-PC has been described in detail elsewhere (Mason et al., 1981a,b). Both phosphatidylcholines were better than 98 mol % pure with regard to the desired positional specificity of the fatty acids on the glycerol backbone. The chemical purity of the phosphatidylcholines was greater than 99 mol %.

Preparation of Sized Multilamellar Liposomal Dispersions. The sized liposomal dispersions were prepared in accordance with the methodology of Olson et al. (1979). The mixed-chain phosphatidylcholines were lyophilized from benzene until constant weight and then dispersed at a concentration of 30 mM in 50 mM KCl prepared from ultrapure KCl (J. T. Baker) and doubly glass-distilled, deionized water. These suspensions were then vortexed for 3–4 min, at room temperature for C(18):C(10)-PC or at 40 °C for C(18):C(14)-PC. The dispersions were then incubated at these temperatures for an additional hour.

So that the liposomes could be sized, 10 mL of the coarse preparation was extruded through a 3-μm polycarbonate membrane filter in a 25-mm holder (Nucleopore, Inc.). This filtration was repeated 2 more times, and a 1-mL sample of the extruded preparation was saved for electron microscopy and high-sensitivity DSC. In this and all subsequent steps, the C(18):C(14)-PC suspensions were heated to 40 °C just prior to extrusion through the membrane filter in order to maintain the samples above the lipid phase transition temperature during filtration. The extruded preparation was then further sized by sequential extrusion through membranes of 1.0-, 0.8-, 0.6-, 0.4-, and 0.2-μm diameter (three filtrations each), and 1 mL of the filtrate was saved from each step in addition to the final 0.2-μm sample.

The phospholipid concentration in each sample was determined by the method of inorganic phosphate (Gomori, 1942) and was subsequently adjusted to 12 mg/mL. We experienced very little change in the lipid concentration during the extrusion procedure, with the concentration of the 0.2-μm preparation always being no less than 26 mM. Finally, the sized preparations were stored at 10 °C under argon and were used for calorimetry or electron microscopy within 24 h. Actually, no change in the size distribution of the preparations was observed by electron microscopy after storage for up to 2 weeks in agreement with the observations of Olson et al. (1979).

Differential Scanning Calorimetry. The calorimetry was performed with a high-sensitivity DSC instrument of the heat conduction type based upon the design of Ross & Goldberg (1974). The construction and operation of this instrument has been described in detail elsewhere (Suurkuusk et al., 1976; Mason et al., 1981a). Scans of both C(18):C(14)-PC and C(18):C(10)-PC dispersions were initiated at 10 °C. All scans were performed at lipid concentrations of 12 mg/mL in excess 50 mM KCl employing a scan rate of 0.26 K min^{-1} in the ascending temperature direction.

Electron Microscopy. Aliquots of coarse or sized dispersions were diluted to 1 mM and spotted onto carbon-coated Formvar grids. A drop of 2% (w/v) ammonium molybdate was added, and excess solution was drawn off with filter paper. The grids were examined with a JEOL-100CX electron microscope. At least 400 liposomes from each sample were photographed (at 6.6K to 33K magnification) and printed at an enlargement of 3 times the negative. Liposome diameters were measured with millimeter calipers and grouped into size intervals of 0.2 μm. For comparison with calorimetry, the distribution of liposomal mass, rather than number, as a function of liposomal size is of principal interest. This distribution was estimated by assuming that the mass of a liposome is proportional to its surfaced area and thus the radius squared. A relative lipid mass term (M_i) for each interval was approximated as $M_i \sim N_i R_i^2$, where N_i is the number of liposomes in interval (i) and R_i is the average radius of that interval. The individual M_i terms were then divided by the sum of the M_i terms to produce the histograms of apparent lipid mass as a function of liposomal size.

Results and Discussion

Mass Distribution of C(18):C(14)-PC and C(18):C(10)-PC Dispersions. The distribution of lipid mass with liposomal size for the C(18):C(14)-PC dispersions is shown in Figure 1. The lipid mass for the unfiltered coarse dispersions (DISP) was distributed among liposomes whose size varied from 0.1 to 2.4 μm with the majority of the lipid mass (75%) falling between 0.1 and 1.2 μm. The mass average liposomal diameter for this population is 1.0 μm. Also shown in Figure 1 are the distribution profiles of C(18):C(14)-PC dispersions sized through 1- and 0.2-μm polycarbonate filters. As can be seen, these filtrations produce a population of liposomes of progressively

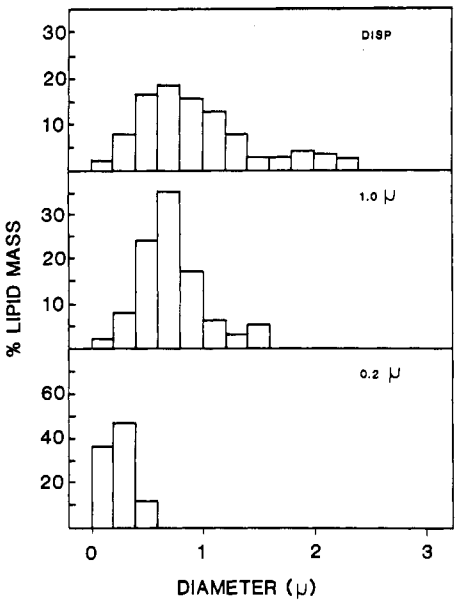

FIGURE 1: Distribution of lipid mass with liposomal size for preparations of C(18):C(14)-PC dispersions. The distribution profiles were determined by negtive-stain electron microscopy as described under Materials and Methods. DISP, unfiltered coarse dispersions; 1.0 μ, dispersion sized through a 1-μm polycarbonate filter; 0.2 μ, dispersions sized through a 0.2-μm polycarbonate filter.

smaller mean diameter and less heterogeneous distribution of lipid mass. This is in agreement with the findings of Olson et al. (1979). The mass average liposomal diameter for the 1- and 0.2μm populations is 0.7 and 0.3 μm, respectively (Table I).

The distribution profiles of the C(18):C(10)-PC dispersions were very similar to those determined for the C(18):C(14)-PC dispersions (data not shown). The unfiltered coarse dispersions were smaller than their C(18):C(14)-PC counterparts with the lipid mass falling between 0.1 and 1.6 μm and 78% of the lipid mass between 0.1 and 0.7 μm. The mass average liposomal diameter is only 0.6 μm. The C(18):C(10)-PC dispersions sized through a 1-μm filter actually showed an increase in the mass average diameter (0.7 μm). This is probably due to the fact that during the filtration, liposomes larger than the pore diameter appear to be broken down and subsequently reassembled near the pore size of the filter (Olson et al., 1979). Thus, the number of liposomes with diameters near 1 μm would be enriched, and this would produce the observed increase in the mean liposomal diameter. Finally, the C(18):C(10)-PC dispersions extruded through a 0.2-μm filter showed a homogeneous distribution of lipid mass with a mean liposomal diameter of 0.3 μm.

It is important to note that the size of the coarse dispersions of C(18):C(14)-PC and C(18):C(10)-PC are considerably smaller than those typically encountered for multilamellar dispersions. For example, the mean diameter of the phosphatidylserine, phosphatidylcholine, and cholesterol (1:4:5) course dispersions analyzed by Olson et al. (1979) was around 1.3 μm, and the range of sizes for dipalmitoylphosphatidylcholine and dimyristoylphosphatidylcholine multilamellar liposomes is reported to be from 0.5 to 5 μm (Marsh et al., 1977).

Calorimetry of C(18):C(14)-PC Dispersions. The main endothermic transition profiles of the C(18):C(14)-PC multilamellar bilayer dispersions are shown in Figure 2, and the corresponding thermodynamic parameters are listed in Table I. The main transition profile of the coarse C(18):C(14)-PC dispersions appears to consist of two highly overlapping

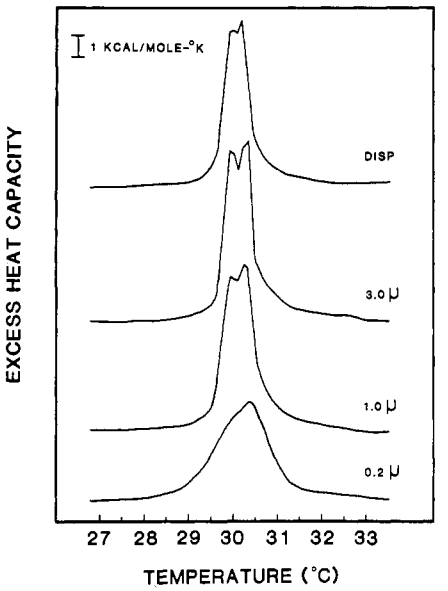

FIGURE 2: Endothermic transition profiles of coarse and sized C-(18):C(14)-PC bilayer dispersions in excess 50 mM KCl. Lipid concentrations of 12 mg/mL and an ascending scan rate of 0.26 K-min^{-1} were employed: see Materials and Methods for details. DISP, unfiltered coarse dispersions; 3.0 μ, dispersions sized through a 3-μm polycarbonate filter; 1.0 μ, dispersions sized through a 1-μm polycarbonate filter; 0.2 μ, dispersions sized through a 0.2-μm polycarbonate filter.

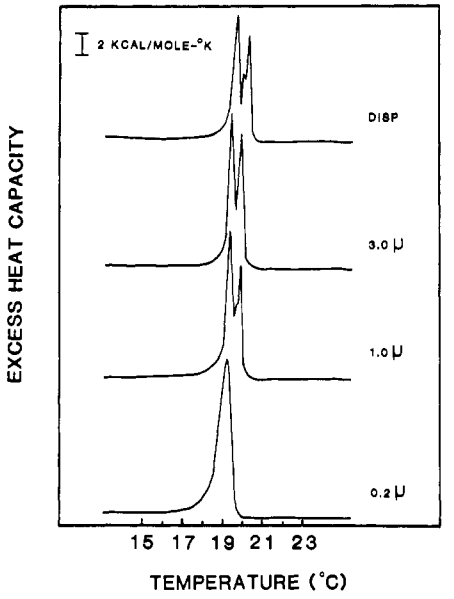

FIGURE 3: Endothermic transition profiles of coarse and sized C-(18):C(10)-PC bilayer dispersions in excess 50 mM KCl. Other conditions are as described for Figure 2.

transition peaks with apparent transition temperatures of 30.0 (T_{m1}) and 30.2 °C (T_{m2}). The transition half-width is 0.63 °C, and the transition enthalpy is 5.5 kcal/mol. These valves are in good agreement with those previously reported (Mason et al., 1981a; Chen & Sturtevant, 1981). As can be seen from Table I and Figure 2, a decrease in the mean size of the C(18):C(14)-PC dispersions is accompanied by an increase in the transition half-width and, with the exception of the 0.2-μm preparation, an increase in transition enthalpy. The resolution of the two overlapping peaks is clearly dependent upon the size of the liposomes, with the 0.2-μm preparation giving the appearance of a single broad peak. The apparent transition temperatures are seen to change very little for the 1- and 3-μm preparations relative to the coarse dispersions.

The 0.2-μm preparation, however, shows an increase in transition temperature to 30.4 °C.

Calorimetry of C(18):C(10)-PC Dispersions. The endothermic transition profiles of the C(18):C(10)-PC dispersions are shown in Figure 3, and the thermodynamic parameters are again listed in Table I. The 0.2-μm preparation consists of a single, although asymmetric, transition peak which is broad (half-width 0.68 °C) and has a large transition enthalpy (12.2 kcal/mol). As the mean size of the liposomes is increased, a second (and possibly a third, T_{m2}) higher temperature peak of very narrow half-width appears. This peak (T_{m3}) can be seen to increase in area relative to the lower temperature peak as the mean size of the liposomes increases. There is also a systematic increase in transition temperature and a decrease in transition enthalpy with an increase in size. For example, the apparent transition temperature of the lower peak (T_{m1}) increases from 19.2 °C for the 0.2-μm preparation to 19.6 °C for the coarse dispersions while the overall transition enthalpy decreases from 12.2 to 9.7 kcal/mol. It should be mentioned that the transition profile of the coarse C(18):C(10)-PC dispersions is of much higher resolution that the one we originally reported (Mason et al., 1981a). The increased resolution reported here is due to improvements in calorimeter instrumentation.

Analysis of the Thermal Phase Transition Cooperativity. The analysis of the transition cooperativity is taken from Marsh et al. (1977) which, in turn, was based upon the Zimm and Bragg theory of cooperative transitions (Zimm & Bragg, 1959). Briefly, it is assumed that the gel ↔ liquid-crystalline phase transition proceeds by the formation of laterally segregated solid and fluid clusters which are surrounded by a perimeter of interfacial phospholipid. At the center of the transition (T_m) the mean size of the solid and fluid regions are equal and experimentally accessible through the cooperativity parameter, σ, as

$$\langle v \rangle = 1/\sqrt{\sigma} + 1 \quad (1)$$

The term $\langle v \rangle$ is the mean size of the cluster regions expressed as the number of cluster molecules per interfacial perimeter molecule. The cooperativity parameter can be obtained as

$$\sigma = (\Delta H/\Delta H_{vh})^2 \quad (2)$$

where ΔH is the calorimetrically determined transition enthalpy (Table I) and ΔH_{vh} is the effective van't Hoff enthalpy of the transition which, in turn, can be approximated as

$$\Delta H_{vh} = \frac{6.9 T_m^2}{\Delta T_{1/2}} \quad (3)$$

where T_m is taken as the temperature of the maximum excess heat capacity and $\Delta T_{1/2}$ is the transition half-width (Mabrey & Sturtevant, 1978).

The quantity $\langle v \rangle$ can also be expressed as

$$\langle v \rangle = (d/f)\langle A/P \rangle \quad (4)$$

where $\langle A/P \rangle$ is the mean ratio of the are of the cluster (A) to the length of the cluster interfacial perimeter (P), f is the area per molecule, and d is the breadth of the perimeter molecules. The physical dimensions of a liposome will impose an upper limit to the transition cooperativity by restricting the size of the fluid and solid clusters developed during the transition. Specifically, at the transition temperature (T_m) each cluster can occupy an area no greater than half the surface area of the liposome. For this restrictive limit, it can be shown that the quantity $\langle A/P \rangle$ becomes equal to the radius of the liposome which we will call the apparent critical radius, R_c. Thus, from eq 1 and 4

$$R_c = (f/d)(1/\sqrt{\sigma} + 1) \quad (5)$$

To determine if liposomal size is limiting the cooperativity of the C(18):C(14)-PC and C(18):C(10)-PC transitions, we must estimate suitable values of f and d. For this purpose we will calculate a lower limit (R_{cl}) and an upper limit (R_{cu}) for the apparent critical radius. A reasonable lower limit for f is the polar head-group area of the phosphatidylcholines in the gel phase as determined by Tardieu et al. (1973). These valves are 41 Å^2 for C(18):C(10)-PC and 48.6 Å^2 for C(16):C(16)-PC (considered here as a contrasting example). For C(18):C(14)-PC the head-group area was estimated to be the average of the values for C(18):C(10)-PC and C(18):C(18)-PC or 46.5 Å^2. For the upper limit of the critical radius, values of f were calculated for the L_α phase according to Büldt et al. (1979). A value of 57 Å^2 was estimated for C(16):C(16)-PC based upon the gel phase head-group area, the relative change in lipid volume through the transition ($\Delta V/V$), and the phosphocholine head-group orientation as determined by neutron diffraction. If we assume that the phosphocholine orientation for C(18):C(14)-PC and C(18):C(10)-PC is unchanged relative to C(16):C(16)-PC and an upper limit for ($\Delta V/V$) for these lipids is that of C(18):C(18)-PC (0.045) as taken from Nagle & Wilkinson (1978), then values of 55 and 48.7 Å^2 are estimated for C(18):C(14)-PC and C(18):C(10)-PC, respectively.

Results from neutron diffraction (Büldt et al., 1979) and ^{31}P NMR (Yeagle et al., 1975, 1976; Gally et al., 1975) indicate that the phosphocholine moiety is predominantly oriented parallel to the bilayer surface in both the ordered and disordered phases. Thus, an estimate for the maximum breadth of the interfacial lipid would be for the phosphatidylcholine molecules to be oriented with their phosphocholine groups parallel to the bilayer surface but tangential to the cluster perimeter. For molecular models, a value of d = 9.6 Å is estimated. Interestingly, this is also the breadth of the B crystal of dimyristoylphosphatidylcholine dihydrate as established by X-ray diffraction (Pearson & Pascher, 1979). The minimum value of d would occur for phosphatidylcholine molecules whose glycerophosphocholine head group and fatty acyl chains are coplanar and oriented normal to the cluster perimeter. From molecular models, a value of d = 4.3 Å is estimated. With the above values, the lower limit of f/d becomes 4.3, 4.8, and 5.1 Å for C(18):C(10)-PC, C(18):C(14)-PC, and C(16):C(16)-PC, respectively, and the upper limit of f/d becomes 11.3, 12.8, and 13.3 Å for C(18):C(10)-PC, (C18):C(14)-PC, and C(16):C(16)-PC, respectively. The values of R_{cl} and R_{cu} calculated from eq 5 and the thermodynamic parameters of Table I are reported in Table I. It is important to discuss the assumptions involved in the analysis of the phase transition cooperativity by the model presented above. First, the critical radius limits for the C(18):C(14)-PC transitions are calculated for the composite transition since the individual endothermic transition peaks are too overlapped to resolve. The values of ΔH and $\Delta T_{1/2}$ employed for the analysis will reflect the mass average contribution of the liposomes comprising each population. However, this seems reasonable since the critical radius limits will be compared to the mass average radius (Table I) of the populations as determined from the mass distribution histograms for the C(18):C(14)-PC dispersions. For the C(18):C(10)-PC coarse dispersions, the limits are calculated with respect to the higher temperature transition peak (T_{m3}; $\Delta T_{1/2}$ = 0.33 °C) by assuming a transition enthalpy of 9.7 kcal/mol, the same as that for the composite transition. As can be seen from eq 2 and 5, this will lead to an underestimate of the

cooperativity of this transition since the transition enthalpy appears to decrease with increasing liposomal size. Also, it should be mentioned that this model must be regarded as qualitative due to the fact that it is a two-dimensional generalization of the one-dimensional Ising model developed by Zim & Bragg (1959) for the helix–coil transition of polymers. However, the model (Marsh et al., 1976, 1977) has been found to give qualitatively similar results to other theoretical analysis of the thermal phase transition of lipid bilayers (Tsong et al., 1977; Freire & Biltonen, 1978). Finally, the mass distribution histograms of the mixed-chain phosphatidylcholine dispersions were calculated by considering only the outer lamella of the multilamellar liposomes. This will clearly result in an overestimate of the mass average radius of the different populations.

For symmetric-chain dipalmitoylphosphatidylcholine coarse dispersions, reasonable bounds for the apparent critical radius of R_{cl} = 0.15 μm to R_{cu} = 0.35 μm can be estimated by employing the thermodynamic data of Mabrey & Sturtevant (1976). Since the size of C(16):C(16)-PC coarse dispersions has been reported to be in the region of 0.5–5 μm, it can be concluded that almost all of the lipid mass will reside in liposomes whose size does not limit the transition cooperativity. This results in a narrow, highly cooperative transition which appears to be first order (Albon & Sturtevant, 1978). This finding is in agreement with the original analysis carried out by Marsh et al. (1977). Recent work on the behavior of fused C(16):C(16)-PC sonicated vesicles (Suurkuusk et al., 1976; Gaber & Sheridan, 1982) and C(16):C(16)-PC large unilamellar vesicles (Takemoto et al., 1981) have indeed shown that liposomes of less than 0.1-μm diameter display thermal transitions which are less cooperative than their multilamellar counterparts.

As shown in Table I, the apparent critical radius for the mixed-chain C(18):C(14)-PC dispersions sized through a 0.2-μm polycarbonate filter can be seen to lie within the range 0.04–0.10 μm. Since the apparent critical radius reflects the mass average contribution of the liposomes within this preparation, it can be concluded from Figure 1 that a minimum of 13–36% of the lipid mass in this preparation will reside in liposomes whose physical size will limit the transition cooperativity as displayed by the composite transition. As the mean size of the C(18):C(14)-PC dispersions is increased, an accompanying increase in the apparent critical radius is clearly seen. For the coarse dispersions the apparent critical radius has increased to between 0.10 and 0.25 μm; however, a minimum of 2–27% of the lipid mass is still found within liposomes whose size is below the apparent critical radius. A similar pattern is observed for the C(18):C(10)-PC dispersions. For C(18):C(10)-PC dispersions sized through a 0.2-μm filter, the apparent critical radius limits are 0.03–0.10 μm which places a minimum of 7–26% of the lipid mass of this preparation within liposomes whose size is below the critical radius. As the mean size of the liposomes is increased, a general trend toward a more cooperative transition is again seen. The apparent critical radius limits for the C(18):C(10)-PC coarse dispersions are 0.10–0.20 μm with respect to the cooperativity of the high temperature endotherm (T_{m3}). This places a minimum of 8–41% of the lipid mass in liposomes whose size is below the apparent critical radius for this transition.

From the above observations, it is evident that for both C(18):C(14)-PC and C(18):C(10)-PC liposomal dispersions an increase in the mean size of the preparations is coupled with an increase in the apparent critical radius as calculated from the cooperativity of the thermal transitions. This leads to the conclusion that the thermotropic behavior of C(18):C(14)-PC and C(18):C(10)-PC dispersions is dictated by the distribution of liposomal sizes within these preparations and the resulting limitation imposed upon the transition cooperativity. The limiting effect of liposomal size is still clearly evident in the coarse dispersions of these mixed-chain phosphatidylcholines. As a result of this effect, information regarding the nature of phospholipid interactions and hydrocarbon chain packing will be largely obscured in the transition endotherms of these lipids.

The detailed shape of the heat capacity profiles of the mixed-chain phosphatidylcholine dispersions will be sensitive to the distribution of liposomal sizes in the preparations for the reasons discussed above and can be understood by considering three observations. First, the above analysis demonstrates that the transition cooperativity is limited in the smaller liposomes present in the dispersions of C(18):C(14)-PC and C(18):C(10)-PC. These liposomes will have a smaller maximal excess heat capacity and larger transition half-width than the larger liposomes of the population where the transition cooperativity is limited to a smaller degree or perhaps not at all. The second observation is that the lipid mass is clearly not homogeneously distributed between large and small liposomes in the coarse dispersions of C(18):C(14)-PC and C(18):C(10)-PC. Third, it is proposed that the conformational order of the acyl chains and perhaps also the phospholipid head groups in the gel phase of the C(18):C(14)-PC and C(18):C(10)-PC bilayers is sensitive to the radius of the curvature of the liposome. As can be seen (Figure 3) there is a subtle but definite increase in transition temperature with increased liposomal size for the endothermic transition peaks of the C(18):C(10)-PC preparations. For C(18):C(14)-PC dispersions, the transition temperature is observed to be slightly higher in the preparation with the smallest liposomes. The consequence of the observations just stated is that the coarse dispersions of C(18):C(14)-PC and C(18):C(10)-PC would be expected to display broad, asymmetric, and possibly multipeaked transition profiles. We now feel that it is the heterogeneous distribution of liposomal sizes which is the source of the apparent multiple peaks and reject the idea that the multiple peaks result from the coexistence of multiple hydrocarbon chain packing phases within these bilayers. The small difference in the apparent transition temperatures observed for the multiple peaks in the transition profiles of these phosphatidylcholines supports this interpretation. If these peaks arose from coexisting domains of interdigitated and randomly packed acyl chains then, as has been previously argued, differences in transition temperatures on the order of 20 °C would be expected (Chen & Sturtevant, 1981; Mason et al., 1981a). Even the relatively small differences in acyl chain packing order that have been demonstrated between sonicated and multilamellar liposomes of the same phosphatidylcholine produce differences in transition temperatures of about 4 °C (Sheetz & Chan, 1972; Gaber & Peticolas, 1977; Gaber & Sheridan, 1982). For the C(18):C(14)-PC and C(18):C(10)-PC dispersions, however, differences in the apparent transition temperatures were always less than 1 °C, suggesting that these peaks do not represent domains of significantly different acyl chain packing configurations. In addition, C(18):C(14)-PC and C(18):C(10)-PC dispersions sized through a 0.2-μm polycarbonate filter, which results in a relatively narrow distribution of liposomal sizes, display transition profiles which are much more symmetric than the corresponding coarse dispersions. Finally, a recently completed Raman spectroscopic investigation of C(18):C(10)-PC multilamellar dispersions reveals a single, sharp transition for this

lipid with the acyl chains in a predominantly all-trans configuration in the gel state (unpublished results). No evidence for multiple packing phases was observed.

In our previous paper (Mason et al., 1981a) it was observed that the C(18):C(10)-PC transition profile was, to some extent, dependent upon the thermal history of the sample. Specifically, the area under the higher temperature transition peak (T_{m3}) appeared to be smaller when the dispersions were cooled slowly (1.5 °C h^{-1}) rather than when they were cooled rapidly (>10 °C h^{-1}) through the phase transition prior to the calorimetric scan. Thus, phospholipid packing in the larger liposomes of C(18):C(10)-PC may be under some degree of kinetic control and subsequently sensitive to thermal history. No dependence on thermal history for the C(18):C(14)-PC transition profile was observed in our study (Mason et al., 1981a); however, some dependence was seen in the study of Chen & Sturtevant (1981).

In our original investigation it was observed that as the acyl chain length inequivalence in mixed-chain phosphatidylcholines was increased beyond a two methylene unit difference, the thermal phase transitions became broader, less cooperative, and generally larger in transition enthalpy. The results obtained here would suggest that this pattern most likely results from a progressive decrease in mean liposomal size as the chain length inequivalence is increased. Consideration of the higher temperature transition peak of the C(18):C(10)-PC coarse dispersions might suggest that for very large liposomes, where the transition cooperativity is not limited, the thermal phase transition remains highly cooperative as the chain length inequivalence is increased. For example, the higher temperature peak (T_{m3}) of the coarse C(18):C(10)-PC dispersions yields a lower limit cooperative unit of 186 molecules. This compares favorably with the corresponding values obtained for C(18):C(16)-PC, 209 molecules, and C(18):C(18)-PC, 141 molecules (Mason et al., 1981a).

A comparison between the mixed-chain phosphatidylcholines examined here and natural or synthetic sphingomyelins (Shipley et al., 1974; Barenholz et al., 1976) is worth consideration. The sphingomyelins, like the mixed-chain phosphatidylcholines, have hydrocarbon tails (the sphingosine chain and acyl chain) which penetrate the bilayer to different depths. Indeed, many of the synthetic sphingomyelins, such as *N*-stearoylsphingosinylphosphocholine and *N*-lignocerylsphingosinylphosphocholine, exhibit broad asymmetric transition endotherms with a large transition enthalpy and low cooperativity reminisceant of the mixed-chain phosphatidylcholines considered here. A recent electron microscopy study of natural sphingomyelin coarse dispersions (Hui et al., 1980) revealed a wide range of liposomal sizes with a surprisingly large proportion of very small, apparently unilamellar, vesicles being present in these preparations. These findings suggest that the thermotropic behavior of many of the sphingomyelins might also be dictated by the distribution of liposomal sizes present in coarse preparations of these lipids. Certainly, the effect of liposomal size must be considered when attempting to explain the thermal behavior of phospholipid bilayer preparations.

Acknowledgments

We thank Anthony V. Broccoli for expert technical assistance. We also acknowledge Sydney Breese and the Central Electron Microscope Facility of the University of Virginia for the electron microscopy.

Registry No. C(18):C(14)-PC, 20664-02-2; C(18):C(10)-PC, 78119-50-3.

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